

The haemoglobin-like protein (HMP) of *Escherichia coli* has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin NADP⁺ reductases

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Received 12 March 1992; revised version received 6 April 1992

Three soluble ferrisiderophore reductases (FsrA, FsrB and FsrC) were detected in *Escherichia coli*. FsrB was purified and identified as the haemoglobin-like protein (HMP) by size and N-terminal sequence analyses. HMP was previously isolated as a dihydropteridine reductase and is now shown to have ferrisiderophore reductase activity. Database searches revealed that the C-terminal region of HMP (FsrB) is homologous to members of a family of flavoprotein oxidoreductases which includes ferredoxin NADP⁺ reductase (FNR). The combination of FNR-like and haemoglobin-like regions in HMP (FsrB) represents a novel pairing of functionally and structurally distinct domains. Structure–function properties of other FNR-like proteins, including LuxG and VanB, are also discussed.

Ferrisiderophore reductase; Haemoglobin; Ferredoxin NADP⁺ reductase; Mosaic protein; LuxG; VanB

1. INTRODUCTION

Iron is a vital constituent of many key cellular components. The biological importance of iron is due, in part, to its ability to fluctuate between two oxidation states (FeII and FeIII). However, owing to the insolubility of FeIII, the amount of free iron in neutral aerated solutions is extremely low. Many bacteria scavenge iron by secreting low molecular weight chelators known as siderophores [1]. Siderophores bind FeIII with high specificity and affinity, forming soluble complexes which are taken up by cognate membrane-associated receptors.

Upon internalisation iron is released from the siderophores, but the mechanism by which this is achieved is poorly understood. It is possible that the complexed iron is reduced to FeII by a ferrisiderophore reductase (FSR). The ferrous iron would then dissociate from the siderophore due to its lower affinity. Indeed, FSRs could be responsible for controlling the absolute and relative levels of intracellular FeII and FeIII.

FSR activity has been detected in a variety of bacteria

and in some cases the enzymes have been isolated [2–4]. However, these enzymes have not been characterised in detail, their role in the cell has not been established, and in most cases the corresponding genes have not been cloned and sequenced.

This paper reports the detection of three discrete and soluble FSR enzymes (FsrA, FsrB and FsrC) in *Escherichia coli*. The purification of one of these enzymes (FsrB) is described and its identity with the haemoglobin-like protein (HMP) of *E. coli* [5] is demonstrated. HMP was previously detected by virtue of its dihydropteridine reductase activity, but the present results indicate that it also has FSR activity. Homology between the C-terminal region of FsrB and members of a family of flavoprotein-oxidoreductases is also reported. The functional and structural implications of these homologies are discussed.

2. EXPERIMENTAL

2.1. Preparation of soluble-cell supernatants

The following *E. coli* K-12 strains were used: JRG2157 (*Δbfr::kan, Δlac-proAB, supE, thi, F⁻*; Timms, A.R., Andrews, S.C., Harrison, P.M. and Guest, J.R. unpublished data) and W3110. Strains were grown in 50 ml L-broth at 37°C. Cells were harvested by centrifugation and sonicated in FSR buffer (60 mM Tris-HCl, pH 8, 30 mM KCl, 30 mM NH₄Cl, 10 mM MgCl₂, 6 mM β-mercaptoethanol) using an MSE Soniprep 150 sonicator at 10 microns for six 10 s pulses with 30 s intervals at 4°C. Soluble cell supernatants were then obtained by centrifugation at 15,000 g for 15 min at 4°C.

Abbreviations: HMP, haemoglobin-like protein; FSR, ferrisiderophore reductase; FNR, ferredoxin NADP⁺ reductase; Fre, flavin reductase enzyme.

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2.2. Electrophoresis and activity staining

Proteins were analysed by polyacrylamide gel electrophoresis (PAGE) [6] under either denaturing conditions (0.1% sodium dodecylsulphate (SDS) in gel and running buffer) or non-denaturing conditions (no SDS). Prior to SDS-PAGE, samples were heated at 100°C for 10 min in loading buffer (0.1 M Tris-HCl, pH 6.8, 10% v/v glycerol, 2.3% w/v SDS, 5% v/v β -mercaptoethanol). Proteins separated by electrophoresis were stained either with silver or with Coomassie blue. FSR activities were detected in native gels using an activity stain [4].

2.3. Purification of FsrB

E. coli K-12 strain W3110 was cultured in two-fold concentrated L-broth at 37°C in a 20 l Biolaftite fermentor for 16 h. Cells were harvested by centrifugation (10,000 \times g for 30 min) and washed by resuspension in FSR buffer followed by centrifugation as before. Cells (66 g) were resuspended in 100 ml of FSR buffer containing 0.04% phenyl methane sulphonyl fluoride and then lysed by two passes through a French pressure cell at 20,000 psi. The whole-cell homogenate was centrifuged at 20,000 \times g for 30 min and the resulting soluble cell supernatant was fractionated by anion-exchange chromatography using a Q-Sepharose FF column (30 \times 20 cm²) equilibrated with FSR buffer. Proteins were eluted using a 1,500 ml linear gradient of 0.03–0.53 M KCl in FSR buffer at a flow rate of 5 ml/min. Fractions containing FsrB were detected after native PAGE by activity staining, and purity was assessed by native- and SDS-PAGE. Active fractions were pooled and concentrated by ultra-filtration in a Filtron Novacell stirred cell (5 kDa nominal molecular weight limit), and then subjected to gel-permeation chromatography using Sephacryl S200-HR (90 \times 5 cm²; FSR buffer, at 30 ml/h). Fractions containing FsrB were precipitated with 50% (w/v) ammonium sulphate and dialysed against FSR buffer. The anion-exchange chromatography step was then repeated and the concentrated fractions were applied to a Phenyl-Sepharose hydrophobicity column (10 \times 1.5 cm²; equilibrated with 1 M (NH₄)₂SO₄ in FSR buffer). The column was washed with distilled H₂O and FsrB was eluted with a 0–100% linear gradient of ethanol at a flow rate of 30 ml/h. The FsrB-containing fractions were precipitated and dialysed and then purified by preparative-PAGE using a 10% T-native polyacrylamide gel. FsrB was located by activity staining, recovered by electroelution, precipitated and purified to homogeneity by repeating the hydrophobicity chromatography.

2.4. Computation

Database searches were carried out using the SEQNET computing facility at the SERC Daresbury laboratory, Daresbury, Warrington. The NEWSWEEP program was used to search the OWL protein database [7,8], FASTA was used to search the NBRF and SWISS-PROT protein databases [9], and TFASTA was used to search the GenBank, EMBL and DAILY nucleic acid sequence databases [9]. The Profile Analysis suite of programs was used both to construct sequence alignments and for protein database searches [10].

2.5 Other methods

FSR activity assays were as described by Fischer et al. [2]. Superose

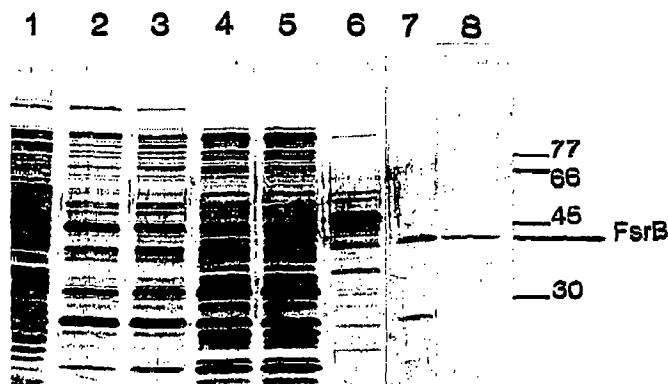


Fig. 1. Purification of FsrB. Silver stained SDS-PAGE (10% T) of samples obtained at different stages of purification (see section 2). Lane 1, soluble-cell extract; lane 2, Q-Sepharose; lane 3, ultrafiltration; lane 4, Sephacryl S200 HR; lane 5, second Q-Sepharose; lane 6, Phenyl-Sepharose; lane 7, preparative-PAGE; lane 8, second Phenyl-Sepharose. The positions and relative molecular masses (10^{-3}) of markers are indicated on the right-hand side.

6 HR (10/30; Pharmacia) and Sephacryl S200-HR (90 \times 5 cm²) columns, each equilibrated in FSR buffer, were used for analytical gel-permeation chromatography. The columns were calibrated using commercial standards (M_r 12,300–700,000, Sigma) and bacterioferritin (M_r 37,000 and 452,000, [11]) as molecular weight markers. Automated solid-phase amino acid sequence determination [12] was performed using a MilliGen/Bioscience 6600 ProSequencer according to the manufacturer's instructions.

3. RESULTS AND DISCUSSION

3.1. Detection of three soluble FSR enzymes in *E. coli*

The soluble proteins from two strains of *E. coli* K-12, W3110 (wild-type) and JRG2157 (Δbfr), were fractionated by native-PAGE and stained for FSR activity. Three active components, denoted FsrA < FsrB < FsrC in the order of their relative mobilities and staining intensities, were detected. There was no significant difference in the relative FSR-staining intensities between the two strains or between samples from exponential and stationary phase cultures, indicating that FSR activity is not influenced by the growth phase or the absence of bacterioferritin. It has previously been ob-

Fig. 2. Multiple sequence alignment of FNR-like domains. Residues thought to be involved in the binding of either FAD or NAD(P)(H) [14] are shown in bold. Secondary structure elements are designated according to Karplus et al. [14]: α -helix (α) and β -strand (β) regions of spinach FNR are indicated above the sequences, and regions that are neither strand nor helix in spinach FNR are indicated with hyphens. Residue numbers are shown at the left and right of each sequence line. Dots in the alignment denote padding characters added to optimise the alignment. The pyridine dinucleotide specificities are indicated. The consensus sequence indicates those regions that are absolutely or partially (<100% and >50%) conserved in upper and lower case, respectively. HMP (FsrB), haemoglobin-like protein from *E. coli* [5]; Fre (FsrC), flavin reductase enzyme from *E. coli* [15]; LuxG, product of the *luxG* gene (possibly a component of the luminescent system) from *V. harveyi* [16]; VanB, vanillate demethylase component B from *Pseudomonas* [17]; XylA, xylene monooxygenase component A from *Pseudomonas putida* [18]; P5, phenol hydroxylase component 5 from *Pseudomonas* [19]; MMO-C, methane mono-oxygenase component C from *Methylococcus capsulatus* [20]; NR, maize nitrate reductase [21,22]; Cb5R, human cytochrome b5 reductase [23]; NOS, rat nitric oxide synthase [25]; SR, sulphite synthase (a subunit) from *E. coli* [26]; CP450R, human cytochrome *P*₄₅₀ reductase [27]; FNR, ferredoxin NADP⁺ reductase from spinach [24].

			S1-1	S1-2	S1-5			
			ββββββββ	ββββββ	βββββ			
Consensus					Gq			
NAD(P)H	HMP	157	IVAKTPRSALITSFEL...	EPVDGGAV.A...	E.YRPGQYLGVW			
NAD(P)H	Fre	4	LSCKVTSVEAITDTVY...	RVRIVPDA.A...	FS.FRAGQYLMVV			
?	LuxG	1	MLCSIEKIEPLTSFIF...	RULLKPDQ.P...	FE.FRAGQYINVS			
NADH	VanB	4	LMIRGLRLLEAPGILGL...	ELVATDGS.P...	LPTFEAGAHLDLH			
NADH	XylA	117	TSALISKQKRLAHDIV...	EMEVVDPK.Q...	IA.FYPGQYADVE			
NAD(P)H	P5	105	YRGVVSALVDLSPTTK...	CLHIKLD.R.P...	MP.FQAGQYVNL			
NADH	MMO-C	115	GLNWVSSNTVQFLLOK...	RPDECGNR.G...	VK.FEPGQFMDLT			
NADH	NR	13	IHCRLVGKKELSRDVR...	LFRFSLPS.PDQVLC	LPICKHIFVCA			
NADH	Cb5R	42	YPLRLIDREIISHDTR...	RFRFALPS.PQHILG	LPVQGHYLSA			
NADPH	NOS	993	SAARLLSRNLQSPKFSRSTIFVRLHTNGNQLQ...	YQPGDHLGVFPGNHEDLVNALIERLEDAPFANHVVKVEMLEERNTALGVISNWKDESR				
NADPH	SR	237	LIATLSVNQKITORNSEKDVRIEIDL	GDSCGLR.YQPCDALGVWYQNDPALVKELVELLWL				
NADPH	CP450R	282	FLAAVTTNRKLNQCTERH.LMHLELDI	SDSKIR.YESGDHVAVYPANDSALVNQLGKILCA				
NADP ⁺	FNR	38	YVGRCLLNTIKITGDDAPGETWHMVFSH	EGE.IP.YREGQSVGVIPDGEDKN				
						S1-4	ββββ	
Consensus							τ ya	
HMP	193						LKPEGFPHQEIRQYS	207
Fre	41						MDERDKRFFS	50
LuxG	38						LSFGSLFFS	46
VanB	42						LPGCLVRPYS	51
XylA	154						CAECSAVRSYS	164
P5	142						LPIDGTRAPS	152
MMO-C	152						IPGTDVSRSSYS	162
NR	54						SIEGKLCMBAT	65
Cb5R	83						RIDGNLVVRPT	94
NOS	1085						LPCTIFQAFKYLDITTPPTPLQQLQFASLATNEKEKQRLVLVSKGLQYEE	1176
SR	313						LP	393
CP450R	368						KHFFPCPTSYRTALTYLDITNPPRTNVLYELAQYASEPSEQLLRKMASSSGEGKELYLSWVVEARRHILAILQDCPSLRPFIHLCCELLPRLQARYYS	467
FNR	87							96
			S1-3	S2-1	S2-2	H-A	S1-6	S3-3
Consensus			β	ββββββββ	βββ	ββββββββ	βββββ	βββββ
HMP	208		as p	1 v	g s yl		vg	p g
Fre	51		LTRKP...	DGK.GYRIAVKRE	EGGQVSNWLNHNAN		VGDDVVKLVAPAGDFFMAV	ADDTFVTLI
LuxG	47		MASTP...	DEKGFIELHIGAS	EINLYAKAVMDRIL		KDHQIVVDIPHGEAYLRD	DQQRPMILI
VanB	52		IASCP...	SNGAFLEWHIGGS	DISKNTLVMBELTN		SWCCGNMVEVSEARGKAWLRD	ESVKPLLV
XylA	185		LCNAP...	GETHYRCLAVLLD	PASRGSSRAVHEQLR		VGQHLTTSAPRNLPLVA	ES.SRSLLF
P5	153		FSAPP...	QPDCLSLFHVRLV	PGGVFSCHLFGGDR		TGATLTTRAPYQGFGLHE	SN.ATMVCV
MMO-C	163		LANFP...	SRNDEVFLHVRV	EGGAATGTIHKQLK		VGDAVELSGPYGQFFVRD	SQAGDLIF
NR	66		PANLP...	NPEGLEFLTRVL	PEGRFSDYLRNDAR		VGQVLSVKGFLGVFGLKE	RCMAPRYFV
Cb5R	95		FTSMV...	DEIGHFDLLVKVYFNE	HPKFPNGGLMTQYLDLP		VGCTIDVKGPLGHVEYTCRGSFVINGKQ	RHASRLAMI
NOS	1177		PISSD...	DDKGFVDLVKIVYFKDT	HPKFFAGGKMSQYLESQ		IGDTIEFRGPGSGLLVYQGGKGFATRDPDKSNFIIRTIVKSVGMI	177
SR	394		ISSSP...	DMYPDEVHLTVAIVSYHTRDGECPVHHGVCSWLNRIQADD			VVPCFVRGA.FSFHLFR	NPQVPCLV
CP450R	468		IASSA...	AEVESEVHIITGVVRYD	IEGRARAGGASSFLADRVEEGGERV		FIEHN.DNFRLLP	NPETPVIMI
FNR	97		IASSS...	KVHPNSVHICAVVVEYET	KAGRINKGVAIWNLRKEPAGENGGRALVPMFVRKS		QFRLPF	KATTPVIMV
			IASSALGDFGDAKSVSLCKVRLITYN	DAGETIKOVCSNFCDLKPGA			EVKLTGFPVCKEMLNPK	DPNATIIML
								168
			H-B	S3-2	H-C	S3-1	H-D	
Consensus			ββ	ββββββββ	ββ	ββββββ	ββββββ	ββββββ
HMP	266		agGtG	p s	1 fg	d	El	s v
Fre	110		SAGVGQTPLMLDLTLA	KA.G	HTAQVNWFAH	AENGDVHFADEVKELGQSLPR	FT	ANTWYRQFSEADRAKQGFDS
LuxG	110		AGGTGFSYARSILLIAL	ARN.P	NRDITIYWG	REEQHY	DLCELEALSILKHP	LQ.VVPVVEQPEA
VanB	111		AGGTGMSYTLSTLKNLS	AQ.G	FNQPIYVYVGA	KDMENLY	VHDELVDIALENKN	VS.YVPVTEISTC
XylA	223		AGGTGITPILAMQVLA	AR.GD	TFELHYCVR	SRRIAADFIDWLEASTFAAHVHLHADDGPTFD		ATALL
P5	212		AGGTGLAPIKCVLQSMTQQA	RE	RDVLLFFGA	QRQDLY	CLDEIEALQLDWGRFE	LIPVLSSESS
MMO-C	222		AGGSGLSPQSMILDL	ER	TRRITLFQ	ARNRAEL	YNCELFEELARHPIS	YVPALNQANDDP
NR	143		AGGTGLAPVSVHVRQEW	APN	ETRIYFGV	NHEPELF	YIDELKSLERSMRN	LT.VKACVWHFSG
Cb5R	178		CGSGGITFMYQIIQAVV	RD.QPED	HTEMHLVYAN	RTEDDIL	LRDELDRWAAEYFPRK	VWYVIDQVKE
NOS	1248		CGGTGIAFRSFWQRRQ	FDIQHGMNP	CPMVLVFGCRQSKIDHI	YREETLQAKNK	GV	FRELYTAYSREPD
SR	459		CGGTGIAFRSFMQRA	AE.G	VEGKNWLFNGNPHFTEDFL	YQVEWQRYVKE	GVLSR	IDLAWSRDQK
CP450R	532		CGGTGIAFRSFIQERA	WL	RQQGKEVGETLLYYGCRSDEYD	YREELAQFHRD	GALTQ	LNVAFSREQS
FNR	169		GTGTCIAFRSFLWKM	F.K	DDYKFKNGLAWFLGV	PTSSSLL	YKEEFK	KEKAPDNFR
			OTGTCIAFRSFLWKM	F.K	DDYKFKNGLAWFLGV	PTSSSLL	YKEEFK	KEKAPDNFR
								LDFAVSREQT
								N.EKGEKM.Y.IQTRMAQY
								254
			H-E	S3-4	H-F	H-G	S3-5	
Consensus			ββββββββ	ββββββββ	ββββββββ	ββββββββ	ββββββββ	ββββββββ
HMP	348		LEGAFSD	PT.MQFYLCGPVGFMTAKQLVDLGVKQE				NHYECFGPHKVL
Fre	198		QDHGTLA	E.HDIYIAGRFE	MAKIARDLFCSEARNAR			EDRLFODAFAT
LuxG	198		SDFRNLS	E.FDIYLCGPYK	MVEVARDWFCDKRAE			PEQLYADAFAYL
VanB	178		RDALRDA	GD.AHLYVCGPGCFMENVLGCARTAGWDET				RLHREYFAAFVQFAGDARAFEGRLARS
XylA	303		KEYLTCQ	P.YEGYLCGPPF	MVDAATELVRLGVAR			ELVFADRFYNRPPC
P5	291		KAHFGGR	FGGQKAYLCGPPF	MIDAATTLTMOGRLE			EDIFMERFYTAADGAGESSRSALFKRI
MMO-C	299		REDLESS	DANFDIYLCGPPC	MIDAACELVRSRGIFC			EQVFFKFLPSGAA
NR	224		REHVPEG	GDDTLALACGPPF	MIQFAISPNLEKMYD			MANSVVF
Cb5R	258		RDHLPPP	EEPLVLNCGPPF	MIQYACLPNLDHVGHP			TERCFVF
NOS	1331		LAESVYALKEQGGHIYVCGDVT	MAADVILKATQIRMTQGGKLEEDAGVFISRLRDDNRHYEDIFGVTLRTYEVNTRLRSESIATFIESKKNDADEVFSS				1429
SR	536		GAELWRWI	NDGAHIYVCGDARRMAADVEKALLEVIAEFGMDLESADYLS	ELRVERRYQDRVY			599
CP450R	622		REHLWKLI	EGGAHIYVCGDARNMARDVQNTFYDIVAELGAMEHAQAVDYIKKLMTKGRYSLDVVS				678
FNR	255		AVELWEMLKKDNTYFYMGSLKG	MEKGIDIMVSLAAEGI				DWIEYKRLKKAQWNEVY
								314

[illegible]

Fig. 3. Alignments for Urf1 and Urf2 of *E. chrysanthemi* with the N- and C-terminal regions of HMP (FsrB). Identities and conservative substitutions (scoring >0.1 in the relatedness odds matrix MDM78 of Dayhoff [13]) are indicated by asterisks and colons, respectively.

served that cellular FSR activity is not dependent on Fur, the ferric uptake regulator [2]. Thus it would appear that FSK activity is not regulated by the level of cellular iron.

3.2. Purification of FsrB and FsrC; identity of FsrB with HMP

The three FSRs were separated into discrete fractions by anion-exchange chromatography, suggesting that they are structurally distinct components. FsrA, FsrB and FsrC represented approximately 7, 23 and 70%, respectively, of the total FSR activity. FsrB was purified to homogeneity, as judged by SDS-PAGE (Fig. 1) and native-PAGE. It exhibited a high affinity for Phenyl-Sepharose and had to be eluted with an ethanol-water gradient. Analytical gel-permeation chromatography and SDS-PAGE with FsrB gave estimated M_r 's of 52,000 and 43,000, respectively, indicating that the protein is monomeric.

A 30-residue N-terminal amino acid sequence was determined for FsrB (data not shown) and found to resemble those of the *Vitreoscilla* haemoglobin-like protein ([28] 69% identity) and other haemoglobins (up to 25% identity) in the OWL database. The N-terminal sequence of FsrB was also found to be identical to that of the haemoglobin-like protein (HMP) of *E. coli*, recently deduced from the *hmp* gene [5]. The HMP of *E. coli* is a monomeric protein (*M_r* 44,000) containing one haem and one FAD [5]. It is therefore concluded that FsrB and HMP are identical.

The FsrC of *E. coli* was partially purified (Shipley, D. and Andrews, S.C, unpublished data) using a procedure similar to that described for FsrB. SDS-PAGE and gel-permeation chromatography revealed that FsrC is a monomer of M_r 26,000. This enzyme probably corresponds to the ferrisiderophore and FAD-reducing enzyme purified previously and designated 'NAD(P)H:flavin oxidoreductase' [29] or 'soluble ferrisiderophore reductase' [2]. The corresponding gene, *fre* (flavin reductase enzyme, Fre) has been cloned and sequenced [15].

3.3. Similarity between HMP (FsrB) and other flavoenzyme oxidoreductases including Fre (FsrC)

Although the N-terminal region (residues 1–140) of HMP (FsrB) is haemoglobin-like, no relationships involving the C-terminal region (residues 141–396) have been reported [5]. Database searches using the complete amino acid sequence of HMP (FsrB) as a probe have now revealed significant similarities between the C-terminal region and the NAD(P)⁺-dependent FAD-binding oxidoreductase domains of proteins in the ferredoxin NADP⁺ reductase (FNR) family, including Fre (FsrC). These similarities are highlighted in the multiple alignment shown in Fig. 2. The three-dimensional structure of one member of the FNR family, spinach FNR, is known [14]. It comprises two structural domains: an FAD-containing domain (residues 19–161) and an NADP⁺-binding domain (residues 162–314). The two domains seem to form a functionally inseparable evolutionary unit, the FNR-functional domain [14]. Residue conservation in the FAD–NAD(P)-binding functional domains of members of the FNR family is particularly

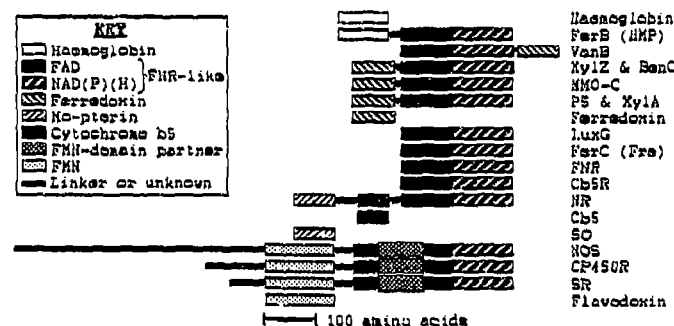


Fig. 4. Domain organisations of members of the FNR family. Schematic representations of the modular relationship between the FNR domain and other linked domains. Abbreviations are as in Fig. 2, except for: BenC, benzoate 1,2-dioxygenase component C from *Achromobacter calcoaceticus* [31]; Cb5, mammalian cytochrome *b*₅ [32]; SO, chicken sulphite oxidase [33]; XylZ, toluate 1,2-dioxygenase component Z from *P. putida* [34].

strong in those regions thought to interact directly with FAD or NAD(P)⁺ [14] (Fig. 2).

Database searches revealed that the LuxG protein [16] from the marine bacterium *Vibrio harveyi* is also a member of the FNR family (Fig. 2), and like Fre does not possess any major structural units other than the FNR domain. The corresponding gene (*luxG*) is part of the luciferase (*lux*) operon. Although the function of LuxG is unknown it may be responsible for the flavin reductase activity providing reduced flavin for the luciferase reaction in many luminous marine bacteria [30]. It is therefore possible that LuxG has a similar role to that of the *E. coli* homologue, Fre (FsrC).

3.4. Putative hmp genes in *Erwinia chrysanthemi* and *Salmonella typhimurium*

Similarities were detected between HMP (FsrB) and two sequences (Urf1 and Urf2) translated from adjacent but different reading frames, located upstream of the *pelA* gene of *Erwinia chrysanthemi* [37]. The sequence of residues 1–57 in Urf1 is 59% identical to the N-terminal region (residues 5–61) of HMP (FsrB) and residues 1–36 of Urf2 are 69% identical to the C-terminal segment of HMP (Fig. 3). These findings suggest that an *E. chrysanthemi* homologue of the *E. coli* hmp gene has suffered a deletion at the junction between the two open reading frames (i.e. between nucleotides 506 and 507 [37]). Interestingly, the 4,978 bp *pel* fragment already contains a deletion in the *pelD* gene at position 2,324 [37].

The database searches also detected an 87 bp segment of the *Salmonella typhimurium* chromosome encoding a sequence that is 100% identical to residues 1–29 of HMP (FsrB). This segment of DNA is presumed to be part of the hmp gene of *S. typhimurium*, located next to the *glyA* gene [38], as in *E. coli* [5].

3.5. Functional and structural implications of the observed similarities

Most members of the FNR family can be described as multi-redox centre proteins because, as illustrated in Fig. 4, they possess flavodoxin-like (FMN-containing), ferredoxin-like ([2Fe-2S]-containing), cytochrome *b₅*-like (haem-containing), Mo-pterin-containing or haemoglobin-like (haem-containing) domains, as well as the FNR domain. The FNR-like proteins containing flavodoxin domains also possess subdomains of approximately 130 residues in the FAD domains which are thought to mediate interaction with the FMN domains [35] (Figs. 2 and 4). The alignment shown in Fig. 2 suggests that these subdomains are in a region corresponding to an external loop between β -strands 1–5 and 1–4.

Those members of the FNR family which possess more than one functional domain are 'mosaic proteins' since they appear to have arisen by the 'shuffling' of domain-encoding regions during evolution. An interest-

ing example of modularization in the FNR family is the VanB protein of *Pseudomonas* [17]. This protein, comprising a ferredoxin-like C-terminal domain and an FNR-like N-terminal domain, forms part of the multi-component vanillate demethylase complex. However, the domain order is reversed in all of the other known examples of partnership between FNR- and ferredoxin-like domains (see BenC, MMO-C, P5, XylA and XylZ in Fig. 4). Nevertheless, the relative locations and orientations of the domains in three-dimensional space could be very similar for VanB and the BenC group of proteins.

The multiple alignment (Fig. 2) indicates that the α -helices and β -strands of FNR are mainly conserved, supporting the view that the proteins have similar structures. However, the region corresponding to an external loop (β -strands 2–1 and 2–2) is apparently missing in the bacterial proteins (HMP, Fre, LuxG, VanB, XylA, P5 and MMO-C; Fig. 2) suggesting that the main chain follows a relatively short path from β -strand 1–3 to helix A.

There are two approximately parallel V-shaped loops at the surface of FNR formed by helices D and E, and helices F and G [14]. The alignment further indicates that nitrate reductase, cytochrome *b₅* reductase and the bacterial proteins lack helix G. This could be accommodated if helix F takes a more direct route from β -strand 3–4 to 3–5. Those proteins which seem to lack helix G also appear to lack the two residues forming the turn between helices D and E (FNR residues 253 and 254) suggesting that helices D and E may be fused to form a single α -helix. If so, the D–E helix could form a more direct link between β -strands 3–1 and 3–4.

The combination of haemoglobin-like and FNR-like domains in HMP (FsrB) represents a novel partnership which raises interesting questions regarding their structure–function relationships. The FNR-like domains are thought to function as flavoprotein reductases passing reducing equivalents between electron acceptors or donors, and pyridine dinucleotides. The FNR-like cytochrome *b₅* reductase participates in methaemoglobin reduction in erythrocytes. It is possible that HMP (FsrB) functions in a similar way such that the FNR-like domain reduces the haem-iron of the haemoglobin-like domain by transferring electrons from NAD(P)H to the haem moiety via the associated FAD group. Alternatively, the haem moiety of HMP (FsrB) may be involved in oxygen-dependent regulation of FsrB activity. The ferric reductase activity of FsrB is probably mediated by flavinonucleotides, as is thought to be the case for FsrC-dependent ferric reduction [15]. HMP (FsrB) also possesses dihydropteridine reductase (DHPR) activity [5], although the HMP (FsrB) studied here is distinct from the previously purified DHPR of *E. coli* [36].

HMP (FsrB) provides an interesting example of the way in which different structural units can be brought together during evolution to generate proteins with

unique structural and functional properties. However, despite the DHPR and FSR activities of HMP (FsrB), the electron acceptor utilised *in vivo*, and therefore the physiological function of the protein, remains uncertain.

Acknowledgements: This work was supported by the Wellcome Trust. We thank the SEQNET service at the SERC Daresbury Laboratory for providing computing facilities, and R.K. Poole and P.J. Artymiuk for helpful discussions.

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